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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The activity of telomerase has been shown to be absent in normal somatic cells, with the exception of stem cells. The reactivation of telomerase has been seen as an early event in most cancers, especially breast cancer. I have previously shown that the inhibition of telomerase led to the inhibition of cell growth via telomere-based mechanisms. During the second year, to understand the mechanism of telomere shortening and growth inhibition, microarray analyses were performed to detect changes in the transcriptional profiling. Also during the second year, another type of telomerase inhibitor, oligonucleotide phosphoramidates, was shown to be an efficient telomerase inhibitor and potential therapeutic agent. Furthermore, I previously showed that telomerase inhibitors and tamoxifen could prevent the spontaneous immortalization of Li-Fraumeni Syndrome-derived breast epithelial cells. During the second year, microarray analyses detected changes in the transcriptional profiling after treatment. Also, preliminary Northern analyses of the spontaneously immortalized breast epithelial cells showed that while the cells were negative for estrogen receptor-alpha (ERα) by histology, the data show that the cells possibly contain ERβ. Tamoxifen mechanism may be to utilize orphan receptors. These studies should lead to new insights in preventing the occurrence or recurrence of breast cancer.

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Introduction

Li-Fraumeni Syndrome (LFS) is characterized by heterozygous mutations in the tumor suppressor gene p53 (1). Alterations in the p53 gene are thought to lead to genomic instability and allow for continued cellular proliferation, leading to further instabilities (2). Studies of the importance of p53, and identification of the LFS germ line mutations of p53, have led to an understanding of the cancer risk to LFS families. Among women in affected LFS families, breast tumors are the most prevalent cancer (afflicting at least 50%), with one quarter of the breast cancers diagnosed before age 30 and 89% diagnosed before age 50 (1,3-4). The molecular mechanism of the specific increased incidence of breast cancer as opposed to other cancers in families affected by LFS is not completely understood (5-6). One of the Li-Fraumeni Syndrome (LFS) cell strains that is used as a model for this project is derived from a patient who had surgery for breast cancer. These human mammary epithelial (HME) LFS cells are telomerase silent, grow in defined medium for approximately 50 population doublings then undergo a crisis stage, reproducibly immortalize and then express telomerase (7).

Cells contain repeated TTAGGG DNA sequences, called telomeres, at the end of chromosomes to provide genomic stability and to provide a source of expendable DNA due to the end replication problem where one DNA strand cannot complete its end during replication (8-10). Telomere length has been shown to decrease with time (and with increasing age). Maintenance of telomeres has been shown to involve telomerase activity, which acts as a reverse transcriptase to add base pairs to the ends of chromosomes (11). It has been proposed that an immortalized cell emerges (a hallmark of most cancer cells) from a stage called crisis when telomerase or another mechanism to maintain telomere stability is activated (12). Once telomerase is activated it stabilizes telomere length and permits continued cell division. Most cancer cells have been shown to contain short but stable telomeres compared to parental cells. Beside germ line and stem cells of renewal tissues, other cells having telomerase activity are cancer cells (90% of those tested) (13).

Telomerase has been shown to become active early in breast cancer progression (13 for review). Thus, the proposed study will determine, using a cell culture model system, if inhibiting telomerase activation has the potential to prevent the occurrence or recurrence of breast cancer. The approach is to examine the effects of telomerase inhibitors, and other putative chemopreventive agents such as tamoxifen, on the growth of immortalized breast epithelial cells and the frequency of spontaneous immortalization in human breast epithelial cell from individuals predisposed to breast cancer.

As telomerase is re-expressed in cancer cells, the critically short telomeres may be favored to be elongated by telomerase. The average telomere length in these cells becomes stable at lengths well below normal cells. These shorter lengths are at a critical length for cell survival. The difference in telomere lengths between normal and cancer cells provides for a mechanism that may specifically target and inhibit the growth of cancer cells. It is proposed that the inhibition of telomerase may lead to the growth inhibition of immortalized human mammary epithelial cells (HMECs) and to the prevention of the spontaneous immortalization of HMECs through telomere shortening.

Body and Conclusions

In the first year, the "Statement of Work's Task 1" was to inhibit the growth of telomerase expressing, spontaneously immortalized Human Mammary Epithelial Cells (HMECs) via a telomere-based mechanism using telomerase inhibitors. I showed that the inhibition of telomerase led to the inhibition of cell growth via telomere-based mechanisms (14, first Annual Report 2001). While non-toxic concentrations of tamoxifen did not inhibit telomerase activity, the 2'-O-meRNA specific to the RNA component of telomerase or the dominant negative hTERT did inhibit telomerase activity. Shortening of telomeres and subsequent growth inhibition followed inhibition of telomerase activity. The method of growth inhibition was determined as apoptotic due to critically shortened telomeres. The next task was to understand the mechanism for the induction of cell death via telomere shortening in these cells. During the second year, microarray analyses were performed to detect any change in the transcriptional profiling after telomerase inhibition. Additional microarray analyses will be performed to verify the preliminary results. Also during the second year, another type of telomerase inhibitor, oligonucleotide phosphoramidates, was shown to be an efficient telomerase inhibitor and potential therapeutic agent (15-16, Appendices A1, A2).

In addition to the tasks outlined for the first year, I was able to show that telomerase inhibitors and tamoxifen can prevent the spontaneous immortalization of Li-Fraumeni Syndrome-derived breast epithelial cells, which have not reached cellular crisis, by the prevention of telomerase activation (17, "Statement of Work's Task 2," First Annual Report 2001). The frequency of spontaneous immortalization of Li-Fraumeni Syndrome HMECs was reduced with tamoxifen and telomerase inhibitors. The finding that treating cells prior to crisis with anti-telomerase agents diminishes the spontaneous immortalization of LFS-derived breast epithelial cells in vitro indicates a potential approach for the development of rational chemoprevention strategies using both clinically and preclinically validated chemopreventive agents for women with a genetic predisposition to breast cancer. Moreover, the prevention of spontaneous immortalization offers a new intermediate endpoint for validating novel chemopreventive agents. The next task was to understand the mechanism for preventing the spontaneous immortalization of these cells. During the second year, microarray analyses were performed to detect any change in the transcriptional profiling after tamoxifen treatment. Additional microarray analyses will be performed to verify the preliminary results. Also, preliminary Northern analyses of the spontaneously immortalized LFS-derived breast epithelial cells showed that while the cells were negative for estrogen receptor-alpha (ERα) by histology, the cells show that they possibly contain ERβ. The method of action by tamoxifen may be to utilize orphan receptors.

Work has begun on the "Statement of Work's Task 3" to understand the fate of breast epithelial cells with different p53 and pRB status when treated with telomerase inhibitors. Cell growth will be monitored and cells will be collected for telomerase activity, telomere length, and protein analyses. Apoptosis and senescence assays will be performed to determine fate of cells. Preliminary data suggest that growth inhibition and apoptosis due to telomerase inhibition is p53 independent. As a side project to understand the role of the p16/Rb pathway in telomere biology and replicative senescence in breast epithelial cells, we showed that inactivation of p16 is not required to immortalize epithelial cells and is a stress response protein (18). Telomere shortening to a critical length may trigger a stress mediated DNA damage response that activates the p16 pathway. While the role of the p16/Rb pathway in telomere biology and replicative

senescence has been understood in human fibroblasts, it has not been understood in epithelial cells (19).

Since cancer is mostly a disease of epithelial cells, we believe our unique system of normal and spontaneously immortalized human breast epithelial cells should provide a good model system to examine the effects of tamoxifen and telomerase inhibitors. Inhibition and/or reversal of the immortal phenotype and other endpoints such as genomic instability, telomere stability, anchorage independent growth assays, should provide insights into the earliest stages of cancer development, leading to more effective cancer prevention measures.

Key Research Accomplishments

Original Statement Task 1. Inhibit the growth of telomerase expressing, spontaneously immortalized Human Mammary Epithelial Cells (HMECs) via a telomere-based mechanism using telomerase inhibitors, Months 1-12:

- The immortalized HMECs were treated with telomerase inhibitors and tamoxifen.
- The lifespan of the treated and untreated cells was monitored and aliquots of cells were either saved for DNA, RNA and protein analyses or stored frozen.
- Cells were lysed and assayed for telomerase activity and telomere length using established laboratory protocols.
- Method of growth inhibition was determined as apoptotic due to critically shortened telomeres.
- Verified that oligonucleotide phosphoramidates are efficient telomerase inhibitors and potential therapeutic agents.
- Microarray data revealed change in transcriptional profiling with telomerase inhibition.

Original Statement Task 2. Prevent the spontaneous immortalization of Li-Fraumeni Syndrome Human Mammary Epithelial Cells using telomerase inhibitors, originally Months 12-24:

- The HMECs were treated with telomerase inhibitors and tamoxifen.
- The lifespan of the treated and untreated cells was monitored and aliquots of cells were either saved for DNA, RNA and protein analyses or stored frozen.
- Approximately ten population doublings before cells enter crisis, fluctuation analyses were performed.
- The frequency of spontaneous immortalization of Li-Fraumeni Syndrome HMECs was reduced with tamoxifen and telomerase inhibitors.
- Microarray data revealed change in transcriptional profiling after tamoxifen treatment.

Original Statement Task 3. Understand the fate of breast epithelial cells with different p53 and pRB status when treated with telomerase inhibitors, Months 24-36:

- The treatment of breast epithelial cells of different p53 and pRB status with telomerase inhibitors is ongoing.
- Monitor cell growth and collect cells for telomerase activity, telomere length, and protein analyses.
- Perform apoptosis and senescence assays to determine fate of cells.

Reportable Outcomes

Publications:

Herbert, B.-S., Wright, W.E., and J.W. Shay. $p16^{INK4a}$ inactivation is not required to immortalize human mammary epithelial cells. *Oncogene*- submitted, 2002.

Ramirez, R.D., **Herbert, B.-S.**, Vaughan M.B., Zou, Y., Gandia, K., Morales, C.P., and J.W. Shay. Bypass of telomere-dependent replicative senescence (M1) upon overexpression of CDK4 in normal human epithelial cells. *Oncogene*- submitted, 2002.

Herbert, B.S., Pongracz, K., Shay, J.W., and S.M. Gryaznov. Oligonucleotide N3'→P5' phosphoramidates as efficient telomerase inhibitors. *Oncogene* 21:638-642, 2002.

Gryaznov, S., Pongracz, K., Matray, T., Schultz, R., Pruzan, R., Aimi, J., Chin, A., Harley, C., **Herbert, B.**, Shay, J., Oshima, Y., Asai, A., and Y. Yamashita. Telomerase inhibitors- Oligonucleotide phosphoramidates as potential therapeutic agents. *Nucleosides, Nucleotides & Nucleic Acids* 20 (4-7):401-410, 2001.

Abstracts:

Steinert, S., **Herbert, B.-S.**, Quinones, H., Wright, W.E., and J.W. Shay. A Breast Cancer Model to Screen Potential Telomerase Inhibitors. Susan G. Komen "Reaching for the Cure...Innovations in Quality Care" Mission Conference. June 1-3, 2002.

Herbert, B.-S., Wright, W.E., and J.W. Shay. $p16^{INK4a}$ inactivation is not required to immortalize human mammary epithelial cells. AACR 93rd Annual Meeting Late-Breaking Abstract. April 6-10, 2002.

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Oligonucleotide N3′→P5′ phosphoramidates as efficient telomerase inhibitors

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Human telomerase is a unique reverse transcriptase that is expressed in multiple cancers, but not in the vast majority of normal cells. The enzyme is responsible for telomere protection and maintenance, and supports the proliferative immortality of cancer cells. Thus, it has been proposed that the specific inhibition of telomerase activity in tumors might have significant and beneficial therapeutic effects. To this goal we have designed, synthesized, and evaluated several oligonucleotide N3′→P5′ phosphoramidates as telomerase inhibitors. These oligonucleotides are complementary to the template region of the RNA domain of telomerase (hTR). The prepared compounds were evaluated in HME50-5E breast epithelial cells, where their effects on telomerase activity were determined using a cell-based telomerase (TRAP) assay at 24 as well as 72 h after exposure to compounds. The oligo-N3′→P5′ phosphoramidate inhibited telomerase activity in cells in the presence of the cellular up-take enhancer (FuGENE6™) in a dose- and sequence-dependent manner, with IC_{50} values of approximately 1 nM. Inhibition of telomerase activity by this compound without the lipid carrier was not efficient. However, the isosequential oligonucleotide N3′→P5′ *thio*-phosphoramidate was able to inhibit telomerase activity with or without lipid carriers at nM, or low-μM concentrations, respectively. This inhibition of telomerase activity in HME50-5E cells by the oligonucleotide *thio*-phosphoramidates was also sequence specific. Long-term treatment of the cells with 0.5 μM of FuGENE6 formulated 13-mer *thio*-phosphoramidates, fully complementary to hTR, resulted in gradual telomere shortening, followed by cellular senescence and apoptosis, as would be predicted for a telomerase inhibitor. The mismatched control compound had no effect on cell proliferation. The results suggest that the oligonucleotide N3′→P5′ phosphoramidates, and particularly *thio*-phosphoramidates, might be further developed as selective anti-telomerase reagents.
Oncogene (2002) **21**, 638–642. DOI: 10.1038/sj/onc/1205064

Keywords: telomerase inhibitors; oligonucleotide; phosphoramidates

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Introduction

Human telomerase is a complex ribonucleoprotein reverse transcriptase composed of an RNA component (hTR) and protein subunit (hTERT). The enzyme is believed responsible for the synthesis of hexanucleotide d-$(TTAGGG)_n$ telomeric repeats at chromosomal ends in dividing cells, employing termini (telomeres) of DNA as primers and a segment of its own RNA as template. Telomerase activity is detected in the vast majority of immortal cell lines, as well as in various primary human tumors. At the same time telomerase activity is not detected in most mature somatic cells (Kim *et al.*, 1994). Cells of constantly renewable tissues and germ line cells are the exceptions, where active telomerase is present during periods of high proliferation. The observed differences in telomerase activity in normal versus tumor derived cells led to the hypothesis that telomerase may represent a suitable target for highly specific anti-cancer therapies. In brief, a treatment with a specific telomerase inhibitor should eventually result in telomere erosion of cancerous cells, and consequently, in their senescence and death. Following this appealing scientific rationale, several classes of telomerase inhibitors were prepared and evaluated. Among the compounds tested were small molecules (Naasani *et al.*, 1998, 1999; Bare *et al.*, 1998; Hisatake *et al.*, 1999), compounds capable of interacting with DNA G-quadruplex structures (Neidle *et al.*, 2000; Hurley *et al.*, 2000), dominant negative hTERT-derived proteins (Hahn *et al.*, 1999), antisense RNA (Feng *et al.*, 1995; Yamaguchi *et al.*, 1999), and various types of oligonucleotides, including DNA phosphodiesters (Glukhov *et al.*, 1998), 2-5A tethered phosphodiesters (Kondo *et al.*, 1998), phosphorothioates (Norton *et al.*, 1996; Matthes and Lehmann, 1999; Sharma *et al.*, 1996), phosphoramidates (Gryaznov *et al.*, 2001), 2′-O-Methyl and 2′-O-(Methoxy-ethyl) phosphorothioate chimera (Pitts and Corey, 1998; Elayadi *et al.*, 2001), ribozymes (Wan *et al.*, 1998), and PNA molecules (Norton *et al.*, 1996). The effects of DNA, PNA and 2′-modified RNA oligonucleotides reportedly were sequence specific, unlike the activity of the majority of earlier generation of phosphorothioates oligomers (Matthes and Lehman, 1999).

In the recently reported crucial proof-of-principle studies, the treatment of various tumor-derived cell

lines with oligonucleotides or protein based telomerase inhibitors resulted in cellular senescence (Herbert *et al.*, 1999; Hahn *et al.*, 1999). The onset of cellular senescence was observed to be in direct correlation with the length of telomeres; cells with relatively short telomeres required much shorter treatments than the ones with longer telomeres (Hahn *et al.*, 1999).

In this study we present our data describing evaluation of oligonucleotide N3′→P5′ phosphoramidates and *thio*-phosphoramidates as telomerase inhibitors *in vivo* in HME50-5E cells (spontaneously immortalized human breast epithelial cell line). The obtained results indicate that these compounds are efficient and sequence-specific telomerase inhibitors with good potential for further development as promising anti-cancer agents.

Results and Discussion

The RNA component of telomerase hTR represents an attractive target for oligonucleotide-based inhibitors. Among other functionally and structurally important regions (Chen *et al.*, 2000), it contains a crucial eleven nucleotides long 'template region', part of which serves as the template for telomere elongation by TTAGGG repeats. Another part of this region may also be involved in chromosomal end recognition by telomerase.

We designed and prepared several 13-mer 2′-deoxyoligonucleotide N3′→P5′ phosphoramidates and *thio*-phosphoramidates (Figure 1), complementary to the template region of hTR. These compounds form very stable, yet sequence-specific duplexes with complementary RNA strands, while remaining highly resistant to hydrolysis by cellular nucleases (Gryaznov, 1999; Pongracz and Gryaznov, 1999). Thus, the melting temperature (T_m) for the complexes formed by the N3′→P5′ phosphoramidate oligonucleotides np-TAGGGTTAGACAA (1) and np-CAGTTAGGGT-TAG (2) with RNA was 72.4°C and 73.0°C respectively, under close to physiological conditions (in PBS

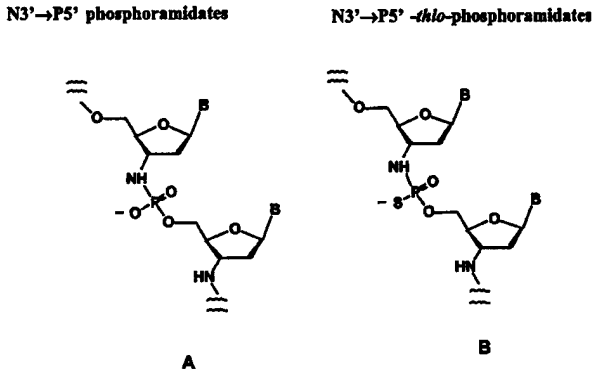

Figure 1 General structure of oligonucleotide N3′→P5′ phosphoramidates – (**a**), and N3′→P5′-*thio*-phosphoramidates – (**b**)

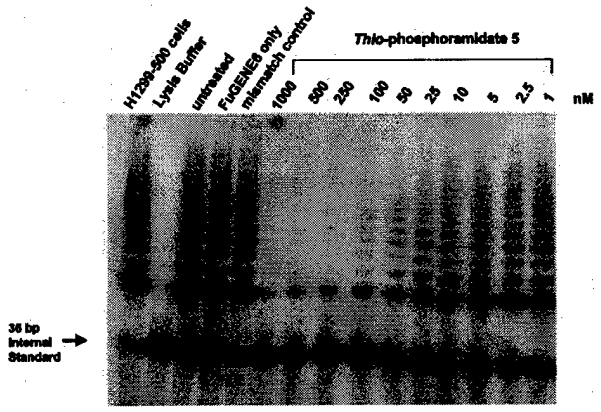

Figure 2 Dose-dependent inhibition activity by *thio*-phosphoramidate oligomer 5 delivered into HME50-5E cells by using FuGENE6 lipid, as measured 24 h post transfection. After transfection, cells were collected and telomerase activity was measured for 500 cell equivalents per lane using a radiolabeled oligonucleotide primer and a polymerase chain reaction (PCR)-based TRAP assay. Labeled PCR reaction products were resolved on polyacrylamide gels and visualized by PhosphorImaging. A ladder of bands represents the extension of the substrate primer by telomerase. Five hundred cell equivalents from H1299 cells served as positive control for the assay. Lysis buffer alone served as a negative control

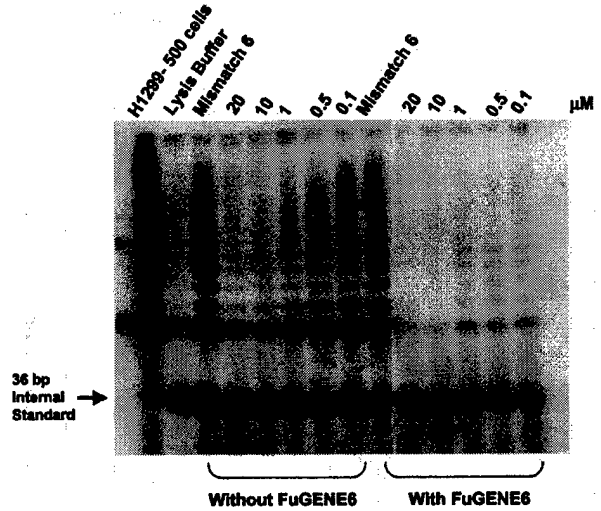

Figure 3 Dose-dependent inhibition activity by *thio*-phosphoramidate oligomer 4 delivered into HME50-5E cells with or without the use of FuGENE6 lipid, as measured 24 h post transfection. After transfection, cells were collected and telomerase activity was measured for 500 cell equivalents per lane using a radiolabeled oligonucleotide primer and a polymerase chain reaction (PCR)-based TEAP assay. Labeled PCR reaction products were resolved on polyacrylamide gels and visualized by PhosphorImaging. A ladder of bands represents the extension of the substrate primer by telomerase. Five hundred cell equivalents from H1299 cells served as positive control for the assay. Lysis buffer alone served as a negative control

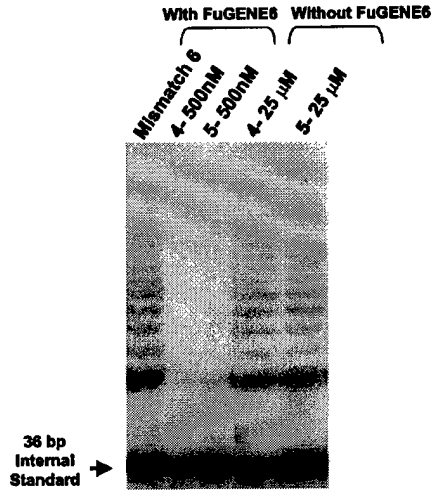

Figure 4 Inhibition of telomerase activity during the long-term treatment by *thio*-phosphoramidates 4 and 5 delivered into HME50-5E cells with or without the use of FuGENE6 lipid, as measured 3 days post transfection. After transfection, cells were collected and telomerase activity was measured for 500 cell equivalents per lane using a radiolabeled oligonucleotide primer and a polymerase chain reaction (PCR)-based TRAP assay. Labeled PCR reaction products were resolved on polyacrylamide gels and visualized by PhosphorImaging. A ladder of bands represents the extension of the substrate primer by telomerase

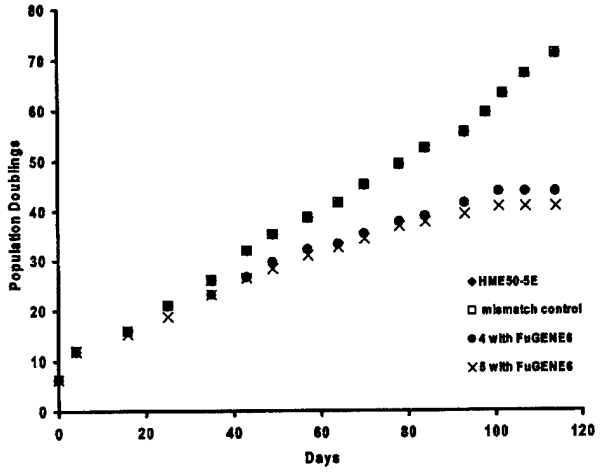

Figure 5 Effects of match 4, 5, or mismatch 6 *thio*-phosphoramidate oligonucleotides (formulated with FuGENE6) on HME50-5E cell growth during long-term treatment experiments. The concentration of all oligonucleotide was 500 nM

buffer). The high thermodynamic stability of complexes formed by these oligonucleotides with RNA, we believe, is crucial for their ability to recognize and bind, via duplex formation to hTR, and consequently to inhibit the enzyme. Another important factor is the position of the targeted site on hTR. It is also important to note that the mismatch control oligonucleotide np-TAGGTGTAAGCAA (mismatched nucleosides are in bold and underlined) (3), having the same nucleoside composition, formed much less stable

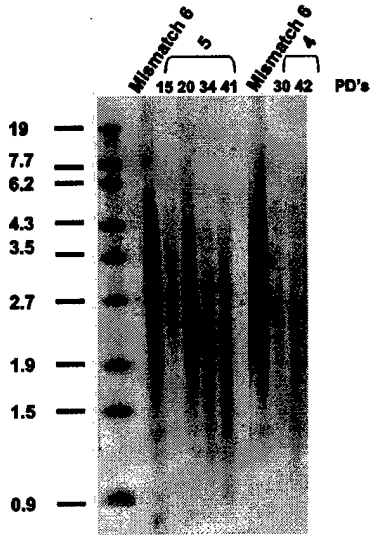

Figure 6 Measurement of telomere restriction fragment length (TRF) in HME50-5E cells treated (long-term, see Figure 5) with match *thio*-phosphoramidate oligomers 4, 5, or mismatch 6. Equivalent amounts of chromosomal DNA were loaded in each lane. The TRF lengths are expressed as kilobase pairs

duplexes with the same RNA strand with T_m value of ~21°C.

The isosequential N3′→P5′ *thio*-phosphoramidate oligonucleotides (NPS) formed duplexes with RNA and demonstrated similar thermal stability – T_m values for nps-TAGGGTTAGACAA, nps-CAGTTAGGGTTAG, and nps-TAGGTGTAAGCAA were 70.0°C, 70.5°C, and <20°C, respectively. These compounds were designated as 4, 5, and 6, respectively.

Following the duplex stability studies, the ability of oligonucleotide N3′→P5′ phosphoramidates to inhibit telomerase activity was evaluated in HME50-5E cells. The cells were exposed to varying concentrations of the compounds in the absence or presence of cationic lipids FuGENE6™. Telomerase activity was determined as a function of the oligonucleotide treatment using a cell-based TRAP assay (Kim *et al.*, 1994) 24 h after exposure to the compounds. The obtained data indicate that hTR complementary oligonucleotide phosphoramidates (NP's) very efficiently inhibited telomerase activity in cells in the presence of the cellular uptake enhancer, with IC_{50} value of ~0.5–1 μM, whereas a mismatched control compound had no effect. In the absence of lipid carrier the anti-telomerase activity of the NP compound was noticeably lower, with IC_{50} value of approximately 20 μM, with mismatched control oligonucleotide not being active at concentrations up to 100 μM (data not shown). Inefficient cellular up-take or intracellular distribution, (such as endosomal sequestration) of lipid un-formulated NP oligonucleotides is likely a key factor reducing the activity of these compounds in cells in the absence of lipid carriers.

We next analysed the effects of telomerase-addressed *thio*-phosphoramidate (NPS) oligonucleotides in HME50-5E cells. The experiments were conducted

similarly to the ones with their NP counterparts. The obtained data demonstrate that NPS oligonucleotides were significantly more efficient telomerase inhibitors, both with and without cellular up-take enhancers, than the parent phosphoramidate compounds. Thus, the IC_{50} values for telomerase inhibition by oligonucleotides 4 and 5 were $\sim$5 nM and $\sim$ 0.5 nM respectively, both in presence of FuGENE6™. At the same time mismatched control NPS compound 6 was not active, with an IC_{50} value much higher than 1000 nM. Importantly, even in absence of lipid carrier NPS oligonucleotides were able to inhibit telomerase in cells; IC_{50} value for compound 4 was approximately 0.5 μM, whereas for the control oligonucleotide 6 it was more than 20 μM. Characteristic gel profiles for cell-based TRAP analysis of telomerase inhibition by NPS oligonucleotides are presented in Figures 2 and 3.

Furthermore, the effects of telomerase-specific NPS oligonucleotides on cellular proliferation were tested in long-term assays. HME50-5E cells were treated with NPS compounds 4, 5 and 6 in the presence or absence of FuGENE6™ at concentrations of 0.5 μM or 25 μM, respectively. The oligonucleotides were added to the cells on every fourth-to-fifth day. Telomerase activity, as well as cellular proliferation (population doublings) was measured during the course of the experiment. The telomerase activity in cells transfected with FuGENE6™ formulated 4 and 5 oligonucleotides was reduced to practically undetectable levels, as determined 3 days post addition of the compounds. Mismatched control 6 again did not affect the enzyme at this concentration (Figure 4). In contrast, un-formulated oligonucleotides exerted a much weaker anti-telomerase effect, even at 50-fold higher doses (Figure 4).

Inhibition of telomerase activity by NPS oligonucleotides 4 and 5 resulted in reduction of the cellular proliferation rate, culminating in the onset of cellular senescence at approximately day 100, followed by massive apoptosis at day 115 of the experiment. At the same time the cells, which were transfected with the mismatched control compound 6, proliferated at the same rate as the untreated counterparts (Figure 5). Moreover, inhibition of telomerase activity by NPS oligonucleotides was accompanied by concomitant reduction in the average telomere length, from $\sim$3 kb to $\sim$1.9 kb, at the time of senescence, as determined by TRF analysis (Figure 6). The length of the shortest telomeric fragments was reduced from $\sim$1.9 kb to $\sim$0.9 kb. The un-formulated (without FuGENE6™) NPS oligonucleotides did not significantly change either cellular proliferation or the length of telomeres in comparison with untreated cells (data not shown). The dramatic difference in the observed effects of the NPS oligonucleotides with versus without lipids is likely due to the significantly lower intracellular or intranuclear concentration of oligonucleotides available for interaction with telomerase in the absence of lipid carrier such as FuGENE6™, resulting in a relatively low level of telomerase inhibition in the cells used. Consequently, we would like to suggest that very high, almost complete level of telomerase inhibition, as was achieved only with lipid formulated NPS oligonucleotides 4 and 5 (see Figure 4), is crucial for induction of telomere shortening and the onset of cellular senescence. In HME50-5E cells, lower levels of telomerase inhibition by un-formulated NPS compounds apparently are not sufficient to cause telomere length reduction.

In summary, oligonucleotide N3′→P5′ phosphoramidates and *thio*-phosphoramidates were designed, prepared, and studied as telomerase inhibitors in cells. The telomerase inhibiting activity of these compounds is highly sequence-specific and dependent on their ability to form stable duplexes with the template region of hTR. The oligonucleotide *thio*-phosphoramidates were significantly more potent telomerase inhibitors than the cognate phosphoramidate compounds, both with or without a cellular up-take enhancer. The NPS oligonucleotides inhibit telomerase activity in immortal HME50-5E cells with IC_{50} values of 0.5–5 nM and 0.5-1 μM with and without lipid carrier FuGENE6™, respectively. Mismatched control oligonucleotides were completely ineffective at the concentrations used. Inhibition of telomerase activity in these cells eventually resulted in a gradual reduction in the length of telomeres, followed by the onset of cellular senescence and apoptosis.

The results obtained warrant further and thorough evaluation of these compounds in *in vivo* and *in vivo* systems as efficient and specific anti-telomerase agents with good therapeutic anti-cancer potential.

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CORRIGENDUM

Oligonucleotide N3′→P5′ phosphoramidates as efficient telomerase inhibitors

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The authors wish to apologise that the name of the first author has been spelled incorrectly. The correct spelling is given above.

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TELOMERASE INHIBITORS – OLIGONUCLEOTIDE PHOSPHORAMIDATES AS POTENTIAL THERAPEUTIC AGENTS

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ABSTRACT

We have designed, synthesized, and evaluated using physical, chemical and biochemical assays various oligonucleotide N3′ → P5′ phosphoramidates, as potential telomerase inhibitors. Among the prepared compounds were 2′-deoxy, 2′-hydroxy, 2′-methoxy, 2′-ribo-fluoro, and 2′-arabino-fluoro oligonucleotide phosphoramidates, as well as novel N3′ → P5′ *thio*-phosphoramidates. The compounds demonstrated sequence specific and dose dependent activity with IC_{50} values in the sub-nM to pM concentration range.

INTRODUCTION

Human telomerase is a complex ribonucleoprotein reverse transcriptase, containing RNA (hTR) and protein (hTERT) subunits. The enzyme is believed responsible mainly for the synthesis of d-$(TTAGGG)_n$ telomeric repeats at chromosomal ends in constantly dividing cells. Telomerase activity is detected in the vast majority of immortal cell lines, as well as in various primary human tumors (1). At the same time telomerase activity is not detected in most somatic cells. Cells of constantly renewable tissues and germ line cells are the exceptions, where active telomerase is

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present. The observed differences in telomerase activity in normal versus tumor derived cells resulted in the hypothesis, that telomerase may represent a suitable target for the specific anti-cancer therapies. Following this appealing scientific rational, several classes of telomerase inhibitors were prepared and evaluated. Among the compounds tested are the small molecules (2), compounds capable of interacting with G-quadruplex structures (3), which telomeres might potentially form, dominant negative hTERT-derived proteins (4), and various types of oligonucleotides, including the phosphodiesters (5), 2–5A tethered phosphodiesters (6), phosphorothioates (7,8), 2′-O-methyl-phosphorothioate chimera (9), ribozymes (10), and PNA molecules addressed to the template region of hTR domain (7). In the recently reported crucial proof-of-principle studies, the treatment of tumor cell lines with the specific oligonucleotides or protein based telomerase inhibitors resulted in cellular senescence (4,11). The onset of cellular senescence was observed to be in direct correlation with the length of telomeres - cells with relatively short telomeres required much shorter treatments, than the ones with longer telomeres (4,11). Based on these initial encouraging results one can infer, that the inhibition of telomerase *in vivo* in cancer cells can lead to attrition of their telomeres, followed by the tumor cells death.

In this study we summarize our results outlining the design, preparation, characterization and biochemical evaluation of several different types of oligonucleotide N3′ → P5′ phosphoramidates as telomerase inhibitors. The obtained results indicate that these compounds are efficient and sequence specific telomerase inhibitors with a good potential for further development as therapeutic agents.

RESULTS AND DISCUSSIONS

The RNA component of telomerase (hTR), which is approximately 455 nucleotide long represents an attractive target for oligonucleotide based anti-telomerase agents. Among other functionally important regions, it contains a crucial eleven nucleotides long so-called template region, part of which serves as the template for telomere elongation by TTAGGG repeats. Another part of this region may also be involved in chromosomal end recognition by telomerase.

First, we designed and prepared several 2′-deoxyoligonucleotide N3′ → P5′ phosphoramidates addressed to the template region of hTR in order to determine optimal oligonucleotide sequence and length requirements – see Figure 1A. The oligonucleotides were synthesized using phosphoramidite transfer methodology on ABI 392 or 394 DNA/RNA automated synthesizers (12,13).

The manufacturer recommended 1μmole DNA synthetic cycle was used, as well as the 3′-aminonucleoside-containing monomer building blocks and CPG-based solid supports, which were purchased from Annovis Inc. The compounds were analyzed and purified by ion exchange (IE) HPLC, and characterized by ^{31}P NMR, mass spectrometry, and by PAGE. The prepared oligonucleotides were

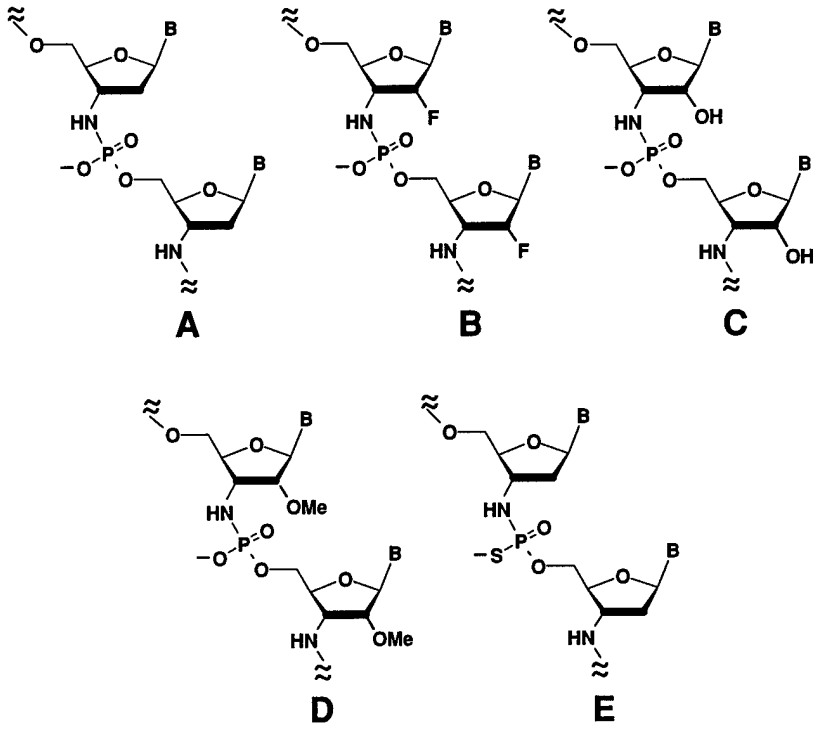

Figure 1. Structure of oligonucleotide N3′ → P5′ phosphoramidates evaluated as telomerase inhibitors.

than evaluated in biochemical assays as telomerase inhibitors, following the determination of the melting temperature (T_m) for their duplexes with the complementary RNA strand. Representative T_m data are presented in Table 3, and the obtained IC_{50} values for telomerase inhibition are summarized in Table 1.

The results indicate that IC_{50} values for the most active compounds reached the sub-nM level. Oligonucleotides with the highest anti-telomerase activity are complementary to a significant part of the hTR template region, particularly to its r-CCC segment. Moreover, anti-telomerase activity of oligonucleotides was highly sequence-dependent. Control compounds with four mismatched bases were practically inactive (more than 1000 times less active than for the fully complementary counterparts) in the assays used. These results are in a good agreement with the corresponding Tm values for the oligonucleotide duplexes: ~72°C and ~21°C, respectively, Table 3. The minimal oligonucleotide length for exerting high anti-telomerase activity is no less than six nucleotides, which also correlates well with the ability of these compounds to form stable duplexes with the RNA target – T_m

Table 1. 2′-Deoxyoligonucleotide N3′ → P5′ Phosphoramidates as Telomerase Inhibitors-Template Region

GRN#	5′-Oligonucleotide-3′	HTR[a]	IC_{50}, nM
	hTR3′-AUGCGGGAAGAGUCAAUCCCAAUCUGUU-5′[b]		
135929	GTTAGGGTTAG	56-46	0.63
135935	GTTGAG TGTAG [c]	56-46	>1000
135993	GUUAGGGUUAG	56-46	0.8
135995	GTTAGGGTTAGAC	56-44	2.4
135998	GTTAGGGTTAGACAA	56-42	1.1
135931	TTAGGG	55-50	10000
135932	TTAGGGTTAG	55-46	2.9
135939	TTAGGGTTAGGG	55-44	2.4
135970	TAGGGTTAGAC	54-44	2.0
135930	TAGGGTTAGACAA	54-42	0.8
135973	TAGGTG TGA ACAA[c]	54-42	>1000
136001	GGGTTAG	52-46	600
136003	GGGuuAG[d]	52-46	280
136000	GGGTTAGAC	52-44	5.9
136012	GGGuuAGAC[d]	52-44	<3.2
135933	AGTTAGGGTTAG	57-46	0.38
135934	CAGTTAGGGTTAG	58-46	0.47
136004	CAGTTAGGGTT	58-48	32
136995	CAGTTAGGG	58-40	32
135971	CCCTTCTCAGTT	65-54	32
136002	CGCCCTTCTCAG	67-56	40
136009	TACGCCCTTCTC	69-58	>1000
136017	CAGuuAGGGuu[d]	58-48	9
136018	GuuAGGGuu[d]	56-48	50

[a]Position of oligonucleotides on hTR; [b]sequence of hTR target, where template region is underlined; [c]mismatched nucleosides are underlined; [d]2′-fluoro-3′-aminonucleosides are depicted as low case u.

for the 2′-deoxy N3′ → P5′ phosphoramidate hexanucleotide TTAGGG is ~36°C under the assay buffer conditions.

Using oligonucleotide phosphoramidates we were also able to identify two other sites on hTR, in addition to the template hTR regions, that were susceptible to the oligonucleotide-induced telomerase inhibition-see Table 2.

The most active non-template addressed oligonucleotide was a 15-mer complementary to nucleotides 137–151 of hTR. It inhibited telomerase activity in the biochemical assays with an IC_{50} value of ~0.4 nM. Other oligonucleotide compounds addressed to the adjacent hTR regions were significantly less active, except the 20-mer GRN 137155, Table 2. It is likely, that the hybridization of the 15-mer oligomer to its hTR target site which, as predicted by molecular modeling, interferes with formation of a pseudo knot structure (14), and results in a significant structural rearrangements of entire hTR molecule. This structural alteration of hTR consequently may lead to the inactivation of the whole telomerase enzyme *via* changing the spatial positioning of hTERT catalytic site

Table 2. 2′-Deoxyoligonucleotide N3′→P5′ Phosphoramidates as Telomerase Inhibitors-Non-Template Regions

GRN#	5′-Oligonucleotide-3′	hTR	IC_{50}, nM
135940	GCTCTAGAATG	166-156	>1000
135941	GCTCTAGAATGAA	166-154	>1000
136006	GCGGCCTGAAAG	367-356	>1000
136007	TCCGTTCCTCTT	382-370	>1000
136008	GCGGGGAGCAAA	92-80	>1000
137159	GTGGAAGGCGGCAGG	151-137	0.42
137158	GCTCTAGAATGAACGGTGGAAGGCGGCAGG	166-137	1
137155	ACATTTTTTGTTTGCTCTAG	179-159	2–10

relative to the template region of hTR, or through rendering the hTR template region partially double stranded and inaccessible to telomere-like DNA primers. The 20-mer GRN 137155, Table 2, designed to hybridize to a predicted stem-loop hTR structure (14), may inhibit telomerase in a similar way. Detailed mechanism of action of non-template region addressed oligonucleotides is currently under further investigation.

Additionally, a second generation of anti-telomerase oligonucleotide N3′ → P5′ phosphoramidates with modifications at the 2′-position of the nucleoside sugar ring was designed and synthesized (12). Evaluation of these 2′-modified phosphoramidate compounds allowed for the assessment of the influence of oligonucleotide sugar-phosphate backbone structure on their activity. Several 11- to 13-mer oligonucleotides, that were isosequential to the most active 2′-deoxy - N3′ → P5′ phosphoramidate counterparts, addressed to the hTR template, were prepared. These modified phosphoramidate oligonucleotides contained 2′-fluoro, 2′-hydroxyl or 2′-O-methyl substitutions–see Figure 1B,C,D. These compounds were assembled and isolated similarly to the 2′-deoxyoligonucleotide phosphoramidates using appropriately protected 3′-amino-5′-phosphoramidite nucleoside building blocks (12). Duplex formation properties of these 2′-substituted oligonucleotide phosphoramidates with RNA strands were evaluated using thermal de-naturation experiments and the corresponding duplex T_m values are summarized in Table 3. Substitution of the oligonucleotide phosphoramidate 2′-hydrogen atom with either a 2′-hydroxyl, (resulting in generation of the natural RNA mimetic), or a 2′-O-methyl, or a 2′-fluoro group results in a noticeable stabilization of the duplexes, formed by these 2′-modified compounds with their RNA complements.

The most thermally stabile duplexes were formed by oligonucleotides containing 2′-fluoro groups. The observed stabilization of the duplexes is likely dictated by the increase in proportion of C3′-endo or N-type of nucleoside sugar puckering for the 2′-substituted oligonucleotide phosphoramidates.

The 2′-modified oligonucleotide phosphoramidates were evaluated as telomerase inhibitors in biochemical assays, and the results are summarized in Table 4. The data obtained indicate, that the activity of studied oligonucleotide

Table 3. Oligonucleotides and Melting Temperatures (T_m) of Their Duplexes with RNA Strands

Expt	5′-Oligonucleotide-3′	Type[a]	T_m,°C	ΔT_m,°C[b]
1	GTTAGGGTTAG	*d/po*	44.2	–
2	TAGGGTTAGACAA	*d/po*	45.2	–
3	GTTAGGGTTAG	*d/np*	72.1	27.9
4	TAGGGTTAGACAA	*d/np*	72.4	27.2
5	TAGG*TG*TA*AG*CAA	*d/np*	~21	−51.3[c]
6	GUUAGGGUUAG	*r/np*	80.0	35.8
7	TAGGGUUAGACAA	*r/np*	82.0	37.8
8	GU^{f}U^{f}AGGGU^{f}U^{f}AG	*2′-F/np*	84.3	40.1
9	GTTAGGGTTAG	*d/nps*	71.5	27.3
10	TAGGGTTAGACAA	*d/nps*	70.0	24.8
11	GTTAGGGTTAG	*2′-OMe/np*	78.2	34.0
12	TAGGGTTAGACAA	*2′-OMe/np*	82.3	37.1

[a]*po, np, 2′-F, nps and 2′-OMe* correspond to phosphodiester, N3′ → P5′ phosphoramidate, 2′-ribofluoro- N3′ → P5′ phosphoramidate, N3′ → P5′thiophosphoramidate, and 2′-OMe- N3′ → P5′ phosphoramidate internucleoside groups, respectively; [b]ΔT_m relative to isosequential natural phosphodiester counterparts; [c]ΔT_m relative to fully complementary compound, exp. 4, and the mismatched nucleosides are underlined.

phosphoramidates is dictated primarily by their ability to hybridize and form stable complexes with their target sites on hTR, and not by the nature of their sugar phosphate backbone. The more DNA-like 2′-deoxy phosphoramidates have similar activity to their more RNA-like 2′-substituted counterparts. Importantly, these

Table 4. Effects of Oligonucleotide Structure on Telomerase Activity

Expt#	5′-Oligonucleotide-3′	Type [a]	IC_{50},nM
1	TAGGGTTAGACAA	*d-np*	0.8
2	TAGGGTTAGACAA	*d-nps*	0.4
3	TAGGGTTAGACAA	*r-np*	0.5
4	TAGGGTTAGACAA	*r-npm*	0.5
5	TAGGGTTAGACAA-Fluor[b]	*d-np*	0.75
6	GTTAGGGTTAG-Fluor[b]	*d-np*	0.3
7	TDGGGTTDGDCDD	*d-np*	0.3
8	TAGG*TG*TA*AG*CAA[c]	*d-np*	>1000
9	TAGG*TG*TA*AG*CAA[c]	*d-nps*	110
10	CAGTTAGGGTTAG	*d-np*	0.6
11	CAGTTAGGGTTAG	*d-nps*	0.05

[a]*np, nps, npm* correspond to N3′→P5′ phosphoramidate, thiophosphoramidate and 2′-OMe-phosphoramidate oligonucleotides respectively; [b]Fluor correspond to fluorescein attached to 3′-aminogroup; [c]mismatched nucleosides are underlined.

results strongly suggest, it is unlikely that the anti-telomerase activity of various types of DNA- and RNA-like oligonucleotide phosphoramidates results from any specific, but sequence independent, high affinity interactions with only the protein component of telomerase - hTERT.

Additionally, a new class of oligonucleotide analogues – oligonucleotide N3′ → P5′ *thio*-phosphoramidates (Fig. 1E) was prepared and evaluated as telomerase inhibitors. These compounds retain high affinity to complementary RNA strands, similar to the parent phosphoramidates, yet are much more acid resistant (15). It was demonstrated before, that the oligonucleotide phosphorothioates are efficient, but highly not sequence specific inhibitors of telomerase (7,16), and that the main site of their interaction with the enzyme was likely the protein hTERT, rather than the RNA component, hTR (8). We decided to combine the attractive properties of two classes of oligonucleotides – high RNA binding affinity of the phosphoramidates and protein interactive capabilities of oligonucleotide phosphorothioates in a single molecule. The structure of oligonucleotide N3′ → P5′ phosphoramidates may allow these compounds to bind in a sequence specific manner to the targeted template region of hTR, and then create, *via* PS-groups-protein contacts, secondary stabilizing interactions with the regions of hTERT in proximity to the hTR hybridized oligonucleotide.

Oligonucleotide N3′ → P5′ *thio*-phosphoramidates were first tested in biochemical assays, and the results are summarized in Table 4. The data demonstrate high and sequence specific telomerase inhibiting activity for the template region addressed *thio*-phosphoramidate oligonucleotides. They were as active, or more active than the isosequential phosphoramidate oligonucleotides. The IC_{50} value for the most potent 13-mer reached ~50 pM, exp. 11, Table 4. Notably, mismatched control compounds were significantly less active (IC_{50} values were 100–250 folds higher), than the fully complementary oligonucleotides. At the same time, the *thio*-phosphoramidate mismatched control compounds were more active telomerase inhibitors, that the isosequential phosphoramidates, but significantly less active then the mismatched or random sequence oligonucleotide phosphorothioates. This would suggest that *thio*-phosphoramidate compounds might have some sequence – independent affinity for hTERT protein, which is in turn significantly lower, than that for the oligonucleotide phosphorothioates.

We next studied the effects of oligonucleotide N3′ → P5′ phosphoramidates on telomerase activity *in vitro* in cells. Various immortal cell lines with different levels of telomerase activity were tested, and telomerase activity was determined as a function of the oligonucleotide concentration, both in presence or absence of lipid-based uptake enhancers. The results are presented in Table 5. A typical gel profile with *in vitro* analysis of inhibition of telomerase activity by oligonucleotides is presented in Figure 2.

The collected data indicate, that oligonucleotide phosphoramidates inhibit telomerase activity in the majority of tested cell lines in a dose and sequence dependent manner. The observed IC_{50} values for telomerase inhibition by lipid

Table 5. IC_{50} Values for *In Vitro* Inhibition of Telomerase by 2′-deoxyoligonucleotide N3′ → P5′ Phosphoramidate–TAGGGTTAGACAA

	IC_{50}, μM^a Lipids	
Cell Type	(−)	(+)
U87	5	nd
293	5	0.1
ACHN	>100	0.3
Caki-1	>100	0.2
PC-3	~30	0.2
A431	>100	0.3
A549	>100	>10
HME50-5E	20	0.5
BJ-hTERT	10	0.5

[a]IC_{50} Value for MM control TAGG*TG*TA*AG*CAA with or without lipids: >100 μM.

carrier-formulated oligonucleotide phosphoramidates are significantly lower, then those for the non-formulated compounds. This may suggest relatively inefficient cellular uptake or unfavorable intracellular distribution of oligonucleotide phosphoramidates in the cell lines used. Importantly, inhibition of telomerase activity in cells by oligonucleotide phosphoramidates resulted in the concomitant reduction in the length of their telomeres, as was expected from the model for the role of telomerase in telomere maintenance.

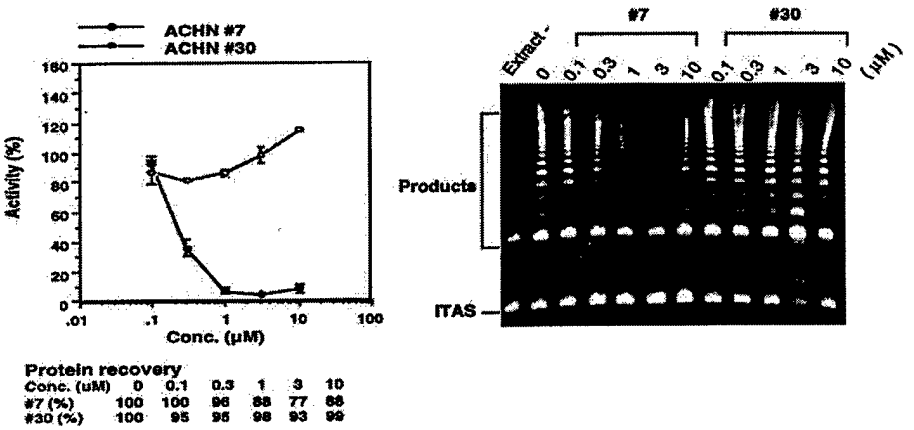

Figure 2. Analysis of telomerase inhibition in ACHN cells treated for 24 hours with Lipofectamine 2000 formulated oligonucleotide phosphoramidates by TRAP assay; #7 and #30 correspond to compounds GRN 135930 and 135973 in **Table 1**, respectively; ITAS is an internal control. Total protein recovery was measured to demonstrate the lack of acute cytotoxicity for the phosphoramidate oligonucleotides.

The obtained results demonstrate that the properly formulated oligonucleotide phosphoramidates can be used as highly efficient and specific telomerase inhibitors in cells. Further studies may identify cell lines where the used phosphoramidates will be more efficacious without the help of uptake enhancers. Moreover, *in vivo* animal models, where cellular uptake and biodistribution might be very different from the oligonucleotide behavior in cells, may serve as better indicators of the therapeutic potential for the telomerase inhibiting phosphoramidates.

Oligonucleotide N3′ → P5′ *thio*-phosphoramidates are currently under *in vitro* and *in vivo* evaluations and the results will be reported in a due course.

In summary, several classes of oligonucleotide N3′ → P5′ phosphoramidates were prepared and studied as telomerase inhibitors. The telomerase inhibiting activity of these compounds is dependent on their ability to form stable duplexes with critical segments of the telomerase RNA component hTR, and not by the nature of their sugar phosphate backbone. The most active oligonucleotides exerted IC_{50} values for sequence specific telomerase inhibition in biochemical assays in the sub-nM to pM range of concentrations, and in cells IC_{50} values were in nM to μM concentration range. The results obtained warrant further development of these compounds as efficient telomerase activity regulating agents with good therapeutic potential.

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